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Enterovirus RNA in Serum Is a Risk Factor for Beta-Cell Autoimmunity and Clinical Type 1 Diabetes: A Prospective Study

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Recent prospective studies have documented serologically an increased frequency of enterovirus infections in prediabetic children, indicating that these infections may initiate and accelerate the beta-cell damaging process several years before the clinical manifestation of type 1 diabetes. The aim of the present study was to establish whether these serological findings would be supported by the detection of enterovirus RNA in a unique prospective series of sera collected from prediabetic children 0–10 years before the manifestation of clinical type 1 diabetes. Reverse transcription followed by polymerase chain reaction employing highly conserved primers among enteroviruses were used to amplify enteroviral sequences. Viral RNA was found in 22% (11/49) of follow-up samples from prediabetic children but in only 2% (2/105) of those from controls (OR 14.9, $P < 0.001$). Persisting RNA positivity was not observed in any of these children. The presence of enterovirus RNA was associated with concomitant increases in the levels of autoantibodies against islet cells (OR 21.7, $P < 0.01$) and glutamic acid decarboxylase (OR 15.4, $P < 0.05$), but not in the levels of antibodies against insulin or the tyrosine phosphatase-like IA-2 protein. In contrast to the prediabetic children, those with newly diagnosed type 1 diabetes were negative for enterovirus RNA. The results thus complement previous serological data, suggesting that enterovirus infections are an important risk factor underlying type 1 diabetes and associated with the induction of beta-cell autoimmunity even years before symptoms appear. ***J. Med. Virol. 61:214–220, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: IDDM; etiology; environmental factors; PCR

INTRODUCTION

Both genetic and environmental factors contribute to the autoimmune process that finally manifests itself as clinical type 1 diabetes. The long subclinical period hampers identification of environmental agents, as they may have been encountered several years before the clinical manifestation of the disease. Prospective studies are thus of major importance.

Among the most strongly suspected environmental agents are enterovirus infections, especially infections caused by coxsackie B viruses (CBV), that have long been linked with type 1 diabetes [Yoon et al., 1979; Barrett-Connor, 1985; Banatvala, 1987]. Previously, when prospective human serial specimens were not available, enterovirus infections were regarded mainly as final precipitating factors in the late course of the

The members of the Childhood Diabetes in Finland (DiMe) Study Group are listed in the Appendix.

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autoimmune process. During the past few years, however, interest has focused increasingly on the enterovirus etiology, as large prospective seroepidemiological studies have implied a role of enteroviruses also in the initiation and acceleration of this process [Hyöty et al., 1995; Dahlquist et al., 1995; Hiltunen et al., 1997]. An increased risk of type 1 diabetes has been associated with infections occurring long before the manifestation of clinical disease, even with infections in utero. These findings have opened up new perspectives for future research, and if the results can be confirmed in new prospective studies, efforts should be made to prevent these infections to reduce the incidence of type 1 diabetes.

Recent reports describing a more frequent occurrence of enterovirus RNA in the serum [Clements et al., 1995] or blood [Andreoletti et al., 1997] of newly diagnosed type 1 diabetes patients than in control subjects have provided further evidence supporting the conception that enterovirus infections are implicated in human type 1 diabetes. In the present prospective study we applied an assay based on reverse transcription and polymerase chain reaction (RT-PCR) to detect enterovirus RNA in the sera of prediabetic children. This is the first study in that the role of enterovirus infections has been prospectively evaluated by direct detection of viral material in serum samples taken during the early stages of the beta-cell-damaging process.

SUBJECTS AND METHODS

Subjects

The subjects were derived from the "Childhood Diabetes in Finland" (DiMe) study, that is described in detail elsewhere [Tuomilehto et al., 1992; Hyöty et al., 1995]. The prospective follow-up sera were collected every 6 months among a cohort of altogether 765 originally non-diabetic siblings of type 1 diabetes index cases. In the present study 93 serum samples from 11 siblings who progressed to type 1 diabetes during the follow-up were examined. The samples had been stored at –20°C for 3 to 10 years. Forty-nine of these samples had never been thawed previously, making them optimal for RT-PCR analysis, whereas 44 had been subjected previously to three to six freezing and thawing procedures. The control group comprised 108 follow-up serum samples from 34 siblings who belonged to the same follow-up cohort but did not develop beta-cell autoimmunity or type 1 diabetes (105 of these samples had never been thawed). The groups of prediabetic and control children were comparable in average age at sample drawing as well as gender distribution. The mean age was 8.4 (range 2.6–17) and 8.9 (range 2.5–20) years and the proportion of males 61% and 60%, respectively. The mean observation period was 4 years in the prediabetic children and 2 years in the controls. The distribution of the observation periods was similar in the two groups within the follow-up years 1987–93.

In addition, serum samples from 47 children with newly diagnosed type 1 diabetes were analyzed. The mean age in this group was 4.4 years and 53% of them were males. None of these samples had been previously thawed.

Reverse Transcription and Polymerase Chain Reaction

RNA was extracted from 140 μl serum samples using a commercial kit (QIAamp viral RNA kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. A primer pair from the highly conserved 5′ noncoding region of the enterovirus genome was used [Hyypiä et al., 1989; Santti et al., 1997] for the RT-PCR. The volume of reverse transcriptase (RT) reaction (40 μl) contained extracted RNA, RT buffer (Promega, Madison, WI), 0.5 mM deoxynucleoside triphosphates (Pharmacia Biotech, Uppsala, Sweden), 4 U of RNase inhibitor (Promega), 50 pmol of the negative-strand primer (5′GAAACACGGACACCCAAAGTA3′) and 20 U of M-MLV reverse transcriptase enzyme (Promega). After incubation for 60 min at 37°C, 10 μl of the cDNA reaction mixture was added to 90 μl of PCR reaction mixture containing PCR buffer (DyNAzyme, Finnzymes, Espoo, Finland), 0.2 mM deoxynucleoside triphosphates (Pharmacia Biotech), 20 pmol of both negative- and positive-strand (5′GGCCCCTGAATGCGGCTAAT3′) primers and 1 U of DNA polymerase enzyme (DyNAzyme). Two drops of mineral oil were added and the tubes incubated in a DNA Thermal Cycler (Perkin-Elmer, Cetus Corp., Norwalk, CT), first for 3 min at 94°C, then through 40 cycles of programmed amplification (denaturation, 94°C, 30 sec; annealing, 53°C, 45 sec; extension, 72°C, 1 min) and finally for 7 min at 72°C. The assay is highly sensitive, as it detects less than 0.1 fg of enterovirus RNA [Santti et al., 1997]. Separate rooms were used in each step of the RT-PCR work, and every analysis included two positive controls (one in RNA extraction and one in RT-PCR) and three negative controls (two RNA extraction blanks and one RT-PCR reagent blank). The RNA-positive samples gave a positive result upon repeated RT-PCR on the first RNA extract.

Hybridization and Sequence Analysis

The PCR amplicons were analyzed first in 2% agarose gels. After electrophoresis DNA was transferred onto a membrane (Gene Screen Plus, Boston, MA) according to the manufacturer's recommendations. Hybridization at 48°C with a digoxigenin-labeled probe (5′CCAAAGTAGTCGGTTCCGC3′) and washes were carried out as recommended for the membrane.

For sequence analysis the PCR products were purified using the QIAEX II DNA Gel Extraction Kit (Qiagen) according to the manufacturer's recommendations. The purified products were cloned by the pGEM-T Vector system I (Promega). Nucleotide sequencing was carried out by the dideoxynucleotide chain-termination method [Sanger et al., 1977; Arola et al., 1996] with universal sequencing primers and modified T7 DNA-polymerase (Sequence Version 2, United States Biochemical Corporation, Cleveland, OH, USA) in the presence of ^{35}S-dATP precursor (Amersham,

Buckinghamshire, UK). The sequence data were analyzed using the Genetics Computer Group software [Devereux et al., 1984]. The PILEUP multiple sequence alignment program was used to generate the dendrograms. Enterovirus sequences described previously used in the comparisons were obtained from the GenBank database.

Autoantibody Analyses

Antibodies against islet cells (ICA), glutamic acid decarboxylase (GADA), insulin (IAA) and the protein tyrosine phosphatase-related IA-2 protein (IA-2A) were analyzed as described previously [Kulmala et al., 1998]. The detection limit of ICA was 2.5 Juvenile Diabetes Foundation units (JDF-U), and tripling of the ICA level was considered a significant increase in antibody level. For GADA, IAA and IA-2A the cut-off limits for antibody positivity were 6.6 relative units (RU), 54 nU/ml and 0.43 RU, respectively. A doubling of the antibody levels was considered a significant increase, but in the case of IA-2A the doubled antibody level had to be at least 1.54 RU. Seroconversion to autoantibody positivity and a significant increase in the levels of autoantibodies were taken as markers of autoimmune activation. The interassay variation in the GADA assay was 18% at an antibody level of 14.6 RU and 12% at levels exceeding 100 RU. The interassay coefficient of variation was less than 8% in the IAA assay, whereas the IA-2A assay showed an interassay variation of 12% at an antibody level of 0.63 RU, 10% at a level of 21.3 RU and 8% at a level of 82.6 RU.

Other Methods

Neutralizing antibodies against CBV serotypes 1–6 were measured using a classical plaque neutralization test [Roivainen et al., 1998]. Viruses were treated with serial fourfold dilutions of sera for 1 hr at 36°C and overnight at room temperature. The serum-treated virus was administered to monolayers of Green monkey kidney cells, and the amount of infectious virus measured by counting the plaques after 46 hr of incubation at 36°C. The reciprocal of the last serum dilution able to block virus infectivity by 80% was taken as the titre of the serum specimen, and a fourfold increase in the titre was considered significant. The HLA-DQB1 alleles associated with type 1 diabetes were determined as previously described [Ilonen et al., 1996]. Odds ratio (OR) with 95% confidence intervals, Fisher's exact test and chi-square test were used in statistical analysis of the results.

RESULTS

None of the 47 serum samples obtained from children with newly diagnosed type 1 diabetes contained enterovirus RNA as determined by RT-PCR. In the case of prediabetic children, however, enterovirus RNA was found in 11 (12%) of the 93 serum samples compared to only two (2%) out of the 108 serum samples from the matched controls (OR 7.1, $P < 0.01$). The RNA-positive samples were taken 0–9.5 years before diagnosis from six prediabetic children, although five prediabetic children were RNA-negative (Fig. 1). None of the RNA-positive children was RNA-positive constantly.

TABLE I. Enterovirus-Associated Risk of Type 1 Diabetes or Induction of Autoantibodies*

	Odds ratio	95% Confidence interval
Type 1 diabetes	14.9	3.2–70.4
ICA	21.7	2.1–224
GADA	15.4	1.4–171
ICA or GADA	15.4	2.4–98.3
IAA	1.2	0.2–6.9
IA-2A	0.9	0.1–8.4

*The Odds ratios refer to risks of induction of autoantibodies at the time of enterovirus RNA positivity, whereas the risk of type 1 diabetes is subsequent to enterovirus RNA positivity. Criteria for significant autoantibody inductions are described in the research design and methods.

Previous thawing of the sera seemed to impair the sensitivity of the RT-PCR, as it was associated with a negative result: no enterovirus RNA was found in the 47 samples thawed previously, whereas 13 out of the 154 not thawed previously gave a positive result ($P < 0.05$). Thus, only the samples, that had not been thawed previously, were included in further analyses. In these optimally stored samples enterovirus RNA was found in 11 out of the 49 samples from prediabetic children (22%) and in two of the 105 samples from the controls (2%) (OR 14.9, $P < 0.001$, Table I).

The occurrence of enterovirus RNA was associated with the appearance or significant increases in ICA and GADA, but not with changes in IAA or IA-2A levels (Table I). According to serological neutralization tests only one of the 11 RNA-positive infections was caused by a CBV serotype. This CBV3 infection was not associated with induction of the autoantibodies, but clinical type 1 diabetes appeared at the time of the infection.

A complete set of follow-up sera was available from one sibling pair. Both of these children had the high risk-associated genotype HLA-DQB1*02/*0302. They were twice positive concomitantly for enterovirus RNA, and at the time of the second infection GADA levels exceeded the cut-off limit for autoantibody positivity in both. They were 3.7 and 12.1 years old at the time of this infection. Only in the younger child were the levels of autoantibodies high and led to a permanent autoimmune process and clinical type 1 diabetes (Fig. 2). Enterovirus RNA was found in altogether four serum samples from this prediabetic child. These four PCR amplicons, as well as the one associated with GADA positivity in her sibling, were chosen for sequence analysis to evaluate the possibility of chronic infection. Based on this analysis the PCR amplicons were taken to originate from the 5′ noncoding region of various non-polio enteroviruses (Fig. 3).

DISCUSSION

Recent large-scale prospective studies have suggested that enterovirus infections could initiate and

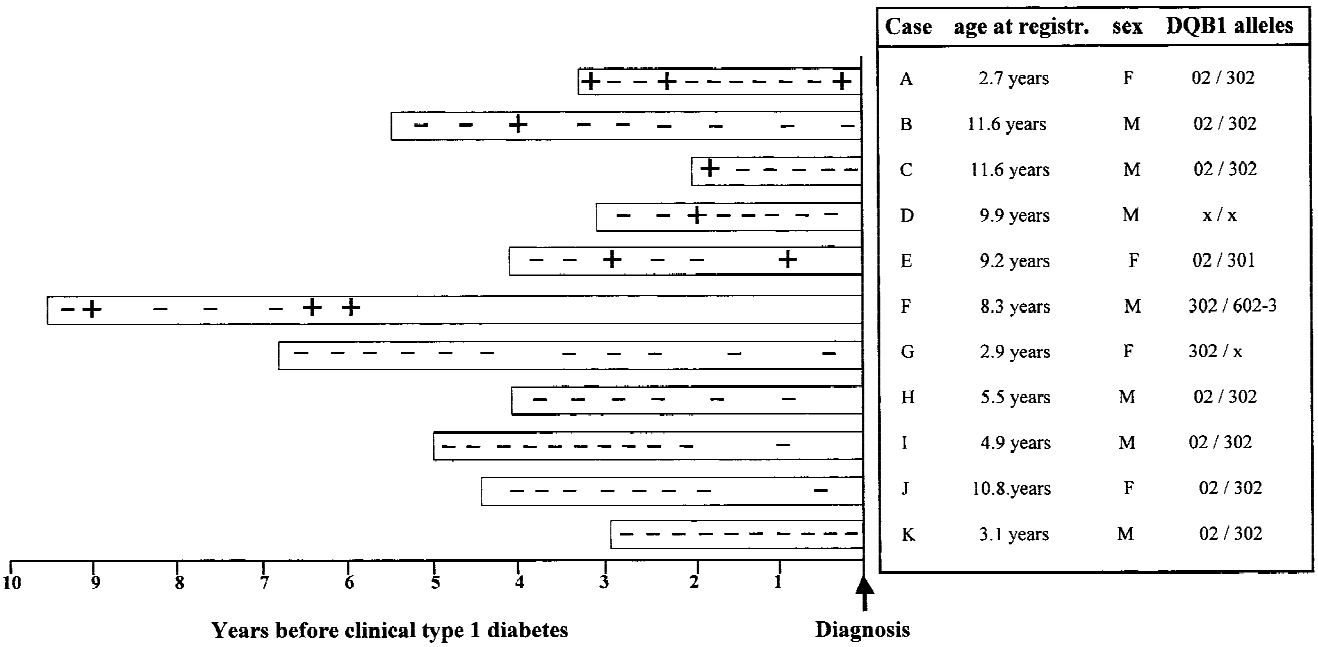

Fig. 1. Prospective follow-up periods of the 11 prediabetic children are indicated by horizontal bars. Serum samples analyzed in the enterovirus RT-PCR assay are marked according to the presence or absence (±) of viral RNA. Age at the beginning of the follow-up period, gender and the type 1 diabetes -related HLA-DQB1 alleles of each child are marked on the right.

accelerate the beta-cell-damaging process years before the manifestation of clinical type 1 diabetes [Hyöty et al., 1995; Dahlquist et al., 1995; Hiltunen et al., 1997;]. In these studies the documentation of enterovirus infections was based on serology. In contrast to serological diagnosis of enterovirus infections, demonstration of enterovirus RNA in serum indicates viremia and hence potential spread of the virus to various organs via the circulation. In view to the existence of highly conserved regions in the enterovirus genome, group-specific RT-PCR assays have been developed [Hyypiä et al., 1989].

Enterovirus RNA was found in 22% of the prospective serum samples taken from prediabetic children but in only 2% of those from control children (OR 14.9, $P < 0.001$). This observation suggests either an increased frequency or a different pathogenesis (prolonged viremia) of enterovirus infections in the prediabetic children, either of that could increase the risk of beta-cell damage. Previous serological data would point to the first alternative, as the frequency of acute enterovirus infections verified serologically was increased in the same prediabetic children [Hyöty et al., 1995].

The results do not support the hypothesis that chronic enterovirus infections precede clinical type 1 diabetes, because persisting enterovirus RNA was not observed in the sera, and sequence analyses indicated that in a child found RNA-positive repeatedly the infections were caused by genetically distinct enteroviruses. The possibility of a local chronic infection of the beta cells cannot be excluded, however, because it is not known whether such an infection could produce sufficient quantities of viruses in the peripheral circulation to be detected by RT-PCR.

The mechanisms by that enterovirus infections could contribute to the process leading to type 1 diabetes are not known. According to the results described above, they could be involved in the induction of autoimmunity against beta-cell antigens, because the occurrence of enterovirus RNA was associated temporally with the appearance of or increases in ICA and GADA levels. This phenomenon was also observed in earlier serological studies [Hyöty et al., 1995; Hiltunen et al., 1997]. The presence of enterovirus RNA in serum was associated with increases in ICA and GADA but not in IAA or IA-2A levels (Table I). As GADA is a part of ICA, the results suggest that enterovirus-induced autoimmunity may be targeted especially against the GAD65 molecule. This is intriguing in that a homologous sequence has been discovered in GAD65 and in the 2C protein of CVB4 [Kaufman et al., 1992]. This sequence is also present in enterovirus serotypes other than CVB4, and it has been shown to be a T-cell epitope both in man [Atkinson et al., 1994] and in the non-obese diabetic mouse [Kaufman et al., 1993], and to induce immunological cross-reactivity between GAD65 and 2C [Lönnrot et al., 1996]. The homologous sequence has also been reported to bind to HLA-DR3 molecules but not to DR4 or DR1 [Vreugdenhil et al., 1998]. Accordingly, HLA-DR3 has been associated with an increased prevalence and high levels of autoantibodies against GAD65 [Sabbah et al., 1996; Genovese et al., 1996]. One may speculate that enterovirus infections could induce an autoimmune response against GAD65 molecules by molecular mimicry in HLA-DR3-positive sub-

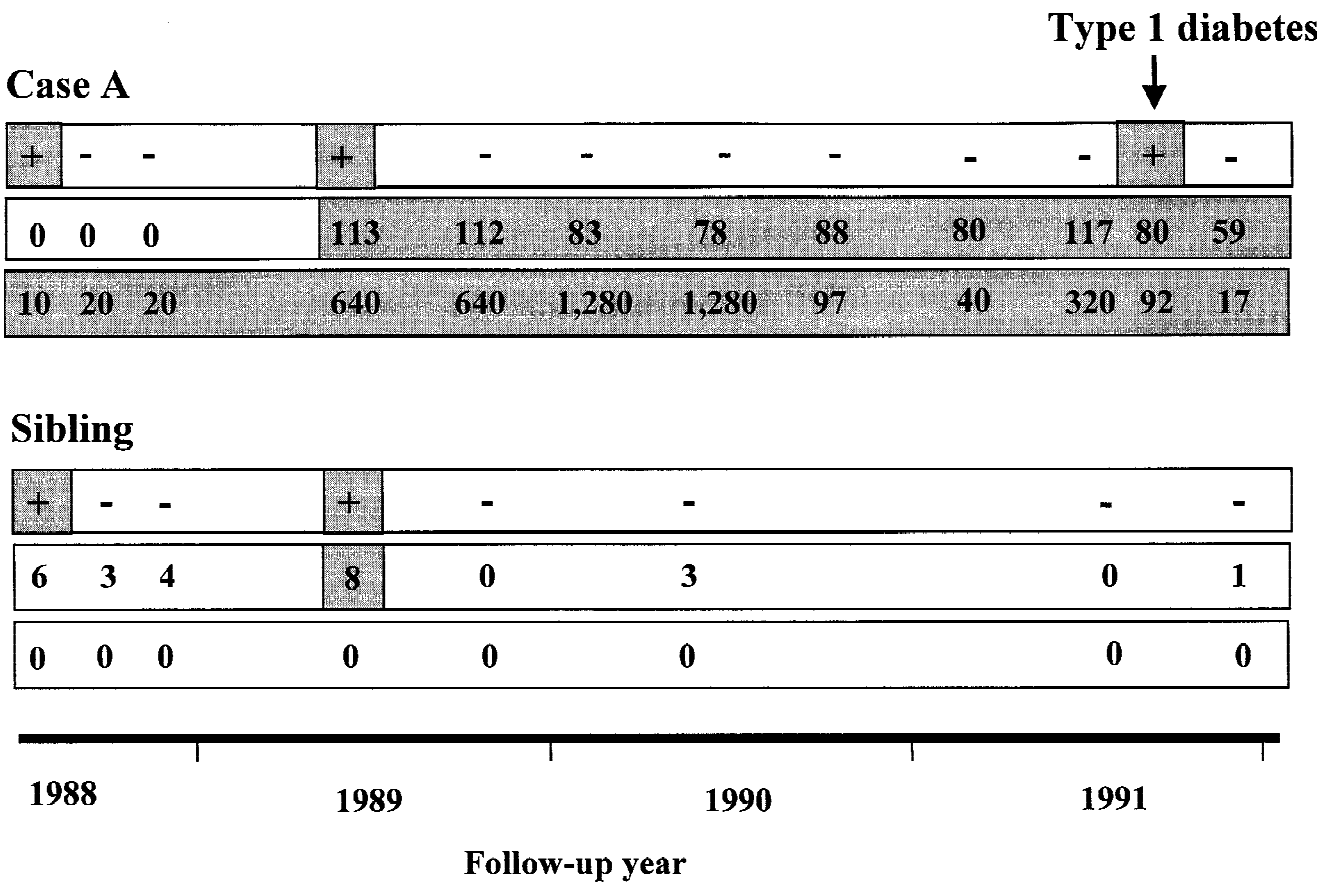

Fig. 2. Presence or absence (±) of enterovirus RNA as well as the levels of ICA (JDF-U) and GADA (RU) are marked in the horizontal bars, that indicate the follow-up period of a sibling pair with the same HLA-DQB1*02/*0302 high-risk alleles. The siblings had two concomitant enterovirus infections and both turned positive for GADA at the time of the second infection. Only in the younger sibling (Case A) was GADA positivity high and persistent, and also ICA levels increased significantly at the time of the infection. In this sibling the autoimmunity led to clinical type 1 diabetes.

jects. In the present study all the children with temporal association between enterovirus infections and induction of GADA carried the DR3-associated DQB1*02 allele, but the number of prediabetic children was too small and the DQB1*02 allele too abundant among them for proper statistical analyses.

The same HLA alleles may mediate susceptibility to both type 1 diabetes and enterovirus infections. If so, then the present results may have been biased by the influence of HLA type, because the case and control groups were not matched for HLA type. The alleles mediating an increased risk of type 1 diabetes were, indeed, more frequent among the case children: e.g., DQB1 haplotype *02/*302 was found in 64% of cases (Fig. 1) vs. 10 % of controls (data not shown). Even if the cases were more prone to viremic enterovirus infections than the controls, however, the temporal relationship between the infections and increases in autoantibody levels can hardly be explained by HLA-related bias.

The present series included a pair of siblings who shared the same HLA-DQB1 high-risk genotype (*02/*0302) and were twice concomitantly enterovirus RNA-positive (Case A and sibling in Figs. 1 and 2). The concomitant presence of viral RNA in serum suggests a spread of the enterovirus within the family. The enterovirus strain that caused the second infection may have been diabetogenic, as both siblings turned positive for GADA at that time. Permanent autoimmunity and type 1 diabetes, however, was induced only in the younger child (age 3.7 years at the second infection). Young age at enterovirus exposure was seen to be linked with the induction of beta-cell autoimmunity in our previous study [Hiltunen et al., 1997], a phenomenon possibly attributable to the immaturity of the immune system of young children.

Among the more than 60 different enterovirus serotypes, CBV serotypes have been most frequently implicated in the pathogenesis of type 1 diabetes [Yoon et al., 1979; Barrett-Connor, 1985; Banatvala, 1987]. A possible role of other serotypes in the pathogenesis, however, has also been indicated [Frisk et al., 1992; Helfand et al., 1995; Roivainen et al., 1998]. The results of the present study support the suggestion than other enteroviruses than CBV could also be capable of inducing beta-cell damage, as only one of the 11 RNA positive infections observed in the prediabetic children was caused by a CBV serotype. Also the sequence

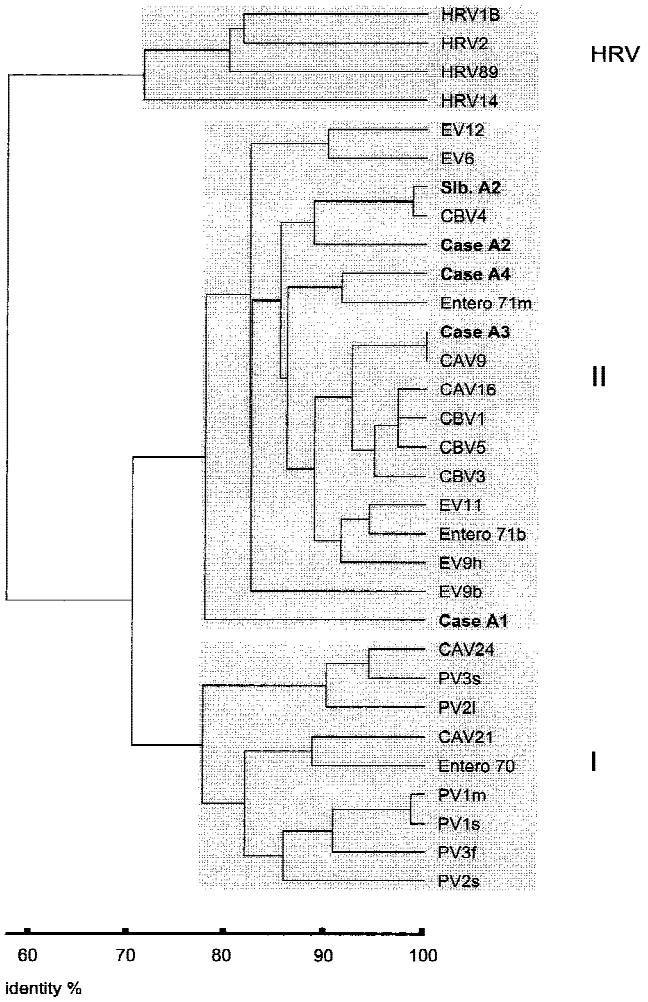

Fig. 3. Dendrogram illustrating the genetic relationships between the amplified sequences and different enterovirus serotypes. The distance on the x-axis increases with sequence dissimilarity. Enteroviruses can be divided into two major genetic groups according to the 5′ noncoding region sequence. These genetic clusters (I and II) are indicated. Comparison with the corresponding region of human rhinoviruses (HRV) is also shown. Case A is a prediabetic child whose four serum samples contained enterovirus RNA. The samples were taken 40 and 28 months before clinical type 1 diabetes (A1,A2), at diagnosis (A3) and one month thereafter (A4). A sibling of Case A also had enterovirus RNA in his serum sample (Sibling A2) taken at the same time as the Case A2 sample. Case A and the sibling are the same as described in Figure 2. HRV, human rhinovirus; CAV, coxsackie A virus; CBV, coxsackie B virus; PV, poliovirus; EV, echovirus.

analyses suggest that the infections were caused by various non-polio enteroviruses, although the genetic conservation of the amplified sequence between enterovirus serotypes hampered specific identification.

None of the 47 children with newly diagnosed type 1 diabetes was enterovirus RNA-positive, and only one of the 11 children observed longitudinally had enterovirus RNA in the serum at the time of diagnosis. These observations support our previous serological data showing no excess of acute enterovirus infections in children at the time of diagnosis of type 1 diabetes [Hyöty et al., 1995]. They are, however, discrepant with other studies indicating a frequent occurrence of enterovirus RNA [Clements et al., 1995; Andreoletti et al., 1997] or increased enterovirus antibody levels in patients with newly diagnosed type 1 diabetes [Barrett-Connor, 1985; Banatvala, 1987; Helfand et al., 1995]. This discrepancy could be due to possible variations in enterovirus epidemiology according to time and place, or to methodological factors, such as the duration of sample storage, the nature of the samples (serum vs. blood) and assay sensitivity.

It is concluded that the present results support previous serological studies suggesting that enterovirus infections are an important risk factor for type 1 diabetes in children even when occurring years before the clinical manifestation of the disease. The findings, based on relatively few subjects, need to be confirmed in larger studies. The ideal setting would be a prospective birth-cohort study with sufficient power for assessment of the etiologic fraction of enteroviruses, identification of the diabetogenic enterovirus serotypes and strains, and evaluation of the underlying pathogenetic mechanisms in human type 1 diabetes.

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APPENDIX

The members of the Childhood Diabetes in Finland (DiMe) Study Group are:

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